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Simple and high-yield purification of urine protein 1 using immunoaffinity chromatography: evidence for the identity of urine protein 1 and human Clara cell 10-kilodalton protein

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ABSTRACT

A simple and high-yield purification procedure for urine protein 1 (UP1) using anti-UP1 immunoaffinity chromatography is described. Pure UP1 was obtained in a final yield of 60.2%, and observed as a single electrophoretic band and as a single peak on reversed-phase high-performance liquid chromatography. The N-terminal amino acid residue of UP1 was found to be glutamic acid, contrary to what was reported previously. Furthermore, the N-terminal sequence of UP1 up to 53 amino acids was confirmed to be identical with that of mature human Clara cell 10-kilodalton protein, which inhibits phospholipase A_2 activity.

INTRODUCTION

Rabbit uteroglobin is a homodimer protein, which consists of identical subunits of 70 amino acids, joined in anti-parallel fashion by two disulphide bridges [1,2]. It is found in the various tissues, such as the endometrium [3], oviduct [4], male genital tract [5], oesophagus [6] and in-lung Clara cells [7]. It has been proposed that the production of this protein is regulated by glucocorticoids and progesterone [8]. This protein binds progesterone [9] and inhibits the activities of several enzymes, such as phospholipase A_2 and papain [10,11]. Furthermore, rabbit uteroglobin is

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supposedly involved in the suppression of the maternal immunological reaction against the foetus [12] and the masking of immunogenicity of male gametes [13].

There are two human proteins that are proposed to be homologous with the rabbit uteroglobin. One is the human Clara cell 10-kilodalton protein (hCC10), which is secreted by Clara cells in the lung [14]. This protein also consists of a dimer of identical subunits of 70 amino acids, which are cleaved from a precursor protein with an additional signal peptide of 21 amino acids. The amino acid sequence of rabbit uteroglobin showed 61% identity with that of mature hCC10 as revealed by cDNA sequencing [15]. Like rabbit uteroglobin, hCC10 inhibits phospholipase A_2 activity [11].

The other protein proposed to be homologous is urine protein 1 (UP1), which Jackson *et al.* [16] originally purified from the urine of patients with renal failure, using a polyclonal antibody produced by DAKO (Copenhagen, Denmark). The molecular mass of UP1 is 14 000–16 000, consisting of two 7000–8000 subunits bound by disulphide bridges. The partial amino acid sequence of UP1 has significant homology with that of rabbit uteroglobin. Furthermore, the amino acid sequence of UP1 is almost identical with that of mature hCC10 except for one difference at the N-terminal residue [16]. Although UP1 has a high degree of homology with rabbit uteroglobin, there have not been any reports on the biological properties of UP1.

In this study, we purified UP1 from pathological urine, using the polyclonal antibody produced by DAKO as an indicator, and determined its amino acid sequence. Furthermore we prepared an anti-UP1 immunoaffinity gel using a monoclonal antibody (mAb) against UP1 in order to establish a simple and rapid purification procedure.

EXPERIMENTAL

Preparation of an anti-retinol binding protein (RBP) polyclonal antibody and immunoaffinity gel

New Zealand white female rabbits were immunized with a total of 2 mg of RBP. The immunoglobulin (Ig) fraction was purified from antisera by 40% ammonium sulphate precipitation. Ig (5 mg/ml) was coupled to an equal volume of the CNBr-activated Sepharose 4B (Pharmacia-LKB, Uppsala, Sweden) according to the method recommended by the manufacturer. The coupling efficiency was estimated to be 98% by measuring the unbound antibody after the coupling reaction.

Preparation of an anti-UP1 mAb and an immunoaffinity gel

Female BALB/c mice were immunized with 50 μg of UP1 emulsified in Freund's complete adjuvant. After four weeks, they received another 50 μg of UP1 and the spleen was removed on the third day. Immune spleen cells were hybridized with mouse myeloma cells (P3-NS1), then subsequently cultured and cloned according to the method of Oi and Herzenberg [17]. Supernatants in each well were tested for binding to UP1 by ELISA. The IgG fraction was purified from ascites fluid by 50% ammonium sulphate precipitation and Sephacryl S-300 column chromatography (Pharmacia-LKB). IgG (5 mg/ml) was coupled to an equal volume of CNBr-activated Sepharose 4B as described above. The coupling efficiency was calculated to be 96% by measuring the unbound antibody.

Purification procedure 1

The starting material was pooled urine from patients with chronic renal failure, whose β_2-microglobulin (β_2m) concentrations were above 15 μg/ml. A 2-l volume of pooled urine was precipitated with 60% ammonium sulphate. The supernatant was further precipitated with 90% ammonium sulphate. The precipitates were dissolved in 10 m*M* Tris–HCl (pH 7.4) containing 0.5 *M* NaCl, and dialysed against the same buffer. Then urine concentrate was applied to a ConA Sepharose-4B column (8.5 cm × 5 cm I.D., Pharmacia-LKB) and non-bound materials, including UP1, were eluted with 10 m*M* acetate buffer (pH 6.5) containing 0.5 *M* NaCl at a flow-rate of 1.0 ml/

min. UP1-containing fractions were pooled and concentrated to 10 ml using a protein concentrator (NM-3, Asahi-kaseikogyo, Tokyo, Japan). The concentrate was loaded onto a Sephadex G-75 superfine column (90 cm × 2.6 cm I.D., Pharmacia-LKB) and eluted with 10 m*M* Tris–HCl (pH 7.4) containing 0.5 *M* NaCl and 0.02% NaN_3 at a flow-rate of 0.3 ml/min. UP1-containing fractions were pooled and concentrated to 10 ml. Thereafter, 5 ml of concentrate were applied to an anti-RBP immunoaffiniy column (10 cm × 2.5 cm I.D.) and non-bound materials, including UP1, were eluted with 10 m*M* Tris–HCl (pH 8.0) containing 0.5 *M* NaCl at a flow-rate of 1.0 ml/min. Bound materials were eluted with 3 *M* NaSCN. This step was repeated twice. UP1-containing fractions were pooled and dialysed against 6.25 m*M* Bis–Tris propane (pH 7.5). After dialysis, 40 ml of the sample were loaded onto a Mono Q HR 5/5 column (Pharmacia-LKB) equilibrated with the dialysis buffer. Proteins were eluted with 50 ml of a 0–100% linear gradient of 0.35 m*M* NaCl in 6.25 m*M* Bis–Tris propane (pH 9.5) at a flow-rate of 0.5 ml/min using fast protein liquid chromatography (Pharmacia-LKB).

Purification procedure 2

Another pooled urine, 3.5 l, was precipitated by the same procedure as described above. After dialysis against 10 m*M* Tris–HCl (pH 7.4), 150 ml of urine concentrate were applied to a Sephadex A-25 column (40 cm × 2.5 cm I.D., Pharmacia-LKB) and non-bound materials were eluted with the above dialysis buffer at a flow-rate of 1.0 ml/min. Bound materials, including UP1, were eluted by a linear gradient of 0–0.3 *M* NaCl in the same buffer, and UP1-containing fractions were pooled. Aliquots (80 ml) were loaded onto an anti-UP1 immunoaffinity column (6 cm × 2.5 cm I.D.), and non-bound materials were removed by washing with 50 ml of 50 m*M* Tris–HCl (pH 8.0) containing 0.5 *M* NaCl. UP1-containing fractions were eluted with 0.2 *M* glycine–HCl (pH 2.0) containing 0.5 *M* NaCl at a flow-rate of 1.0 ml/min, pooled and dialysed against 100 m*M* Tris–HCl (pH 7.4). A reversed-phase column (μBondasphere 5 μm C4-300A, 150 mm × 3.9 mm I.D., Waters, Milford, MA, USA) was connected to an HPLC instrument (Toso, Tokyo, Japan) and equilibrated with 0.05% trifluoroacetic acid (TFA) in distilled water. Aliquots (12 ml) were applied to the column and eluted with a 0–100% linear gradient of acetonitrile in 0.05% TFA at a flow-rate of 1.0 ml/min. UP1-containing fractions were lyophilized and redissolved in 1 ml of distilled water.

Protein measurement, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting analysis

The protein concentration was determined using the Coomassie protein assay reagent (Pierce, Rockford, IL, USA), with bovine serum albumin (BSA) as the standard. SDS-PAGE was performed according to the method of Schägger and Jagow [18] using a 10% separation gel. The gel was stained with Coomassie Brilliant Blue R-250. This SDS-PAGE system used tricine as a trailing ion, which allowed a good resolution of small proteins. The 7000-dalton position was determined using a calibration curve (data not shown).

Proteins separated by SDS-PAGE were transferred onto a membrane according to the method of Towbin *et al.* [19]. Immunostaining with anti-UP1 antibody (Ab) was carried out using either mouse monoclonal anti-UP1 Ab (1 μg/ml) or rabbit polyclonal anti-UP1 Ab [DAKO, diluted 1:500 with 1% BSA–phosphate-buffered saline (PBS)] as a first Ab and horseradish peroxidase (HRP)-conjugated goat either anti-mouse IgG Ab (American Qualex, La Mirada, CA, USA, diluted 1:1000 with 1% BSA–PBS) or anti-rabbit IgG Ab (American Qualex, diluted 1:1000 with 1% BSA–PBS) as a second Ab, respectively. Immunostaining with anti-RBP Ab was performed using rabbit polyclonal anti-RBP Ab (DAKO, diluted 1:500 with 1% BSA–PBS) as a first Ab and HRP-conjugated goat anti-rabbit IgG Ab (American Qualex, diluted 1:1000 with 1% BSA–PBS) as a second Ab. Bound antibodies were detected using a Konica immunostaining HRP kit (Konica, Tokyo, Japan), following the manufacturer's instructions.

Enzyme-linked immunosorbent assay (ELISA) for UP1

The level of UP1 at each purification step was determined by a sandwich ELISA. ELISA plates were coated with 100 μl of anti-UP1 mAb (1 μg/ml in 50 m*M* carbonate buffer (pH 9.6) overnight at 4°C. After washing with PBS containing 0.05% Tween-20, these plates were coated with 200 μl of 1% BSA–PBS. The plates were washed a further three times, and 100 μl of various dilutions of samples were added and incubated for 90 min at 37°C. After the plates had been washed, 100 μl of rabbit polyclonal anti-UP1 Ab (DAKO, diluted 1:1000 with 1% BSA–PBS) were added and incubated for 90 min at 37°C. Subsequently, the plates were washed, supplemented with 100 μl of HRP-conjugated goat anti-rabbit IgG Ab (American Qualex, diluted 1:2000 with 1% BSA–PBS) and incubated for 40 min at 37°C. Finally, 100 μl of enzyme substrate [1 mg/ml *o*-phenylenediamine in citric buffer (pH 5.0) containing 0.03% hydrogen peroxide] were added. After incubation for 6 min, the enzyme activity was stopped by adding 150 μl of 1.0 *M* H_2SO_4, and the absorbance at 492 nm was measured.

Cross-reactivity with other urinary proteins, such as human IgG, human albumin, RBP, α_1-microglobulin (α_1m) and β_2m, was also investigated. RBP, α_1m and β_2m were purified according to methods described previously [20–22]. Human IgG was purchased from Serva (Heidelberg, Germany) and human albumin from Sigma (St. Louis, MO, USA). Serial dilutions of the above proteins from 100 to 0.1 μg/ml were assayed in this system.

Amino acid sequencing

UP1, purified by reversed-phase HPLC, was loaded onto a 470A gas-phase amino acid sequencer connected to a 120A-900A PTH-amino acid analysing system (Applied Biosystems, Foster City, CA, USA) and analysed according to the manufacturer's instructions.

RESULTS

Purification procedure 1

Because Jackson *et al.* [16] reported that the molecular mass of UP1 was *ca.* 14 000–16 000, we expected that patients with proximal tubular impairment would excrete large amounts of UP1 as well as other low-molecular-mass proteins, including β_2m, α_1m and RBP. As starting materials, we collected urine with a β_2m concentration of more than 15 μg/ml. After ammonium sulphate precipitation, UP1 was detected between 60% supernatant and 90% precipitation. Because we observed that UP1 did not bind to Con-A, glycoproteins were removed from the UP1-containing fraction by ConA affinity chromatography (Fig. 1, lane 3). Samples were then loaded onto a Sephadex G-75 column, which removed most proteins with a molecular mass of above 30 000. RBP (indicated by a filled triangle in Fig. 1, lane 4), β_2m and other unidentified proteins, however, still remained at this stage (Fig. 1, lane 4). The concentration of RBP in this fraction was calculated to be more than 400 μg/ml, because

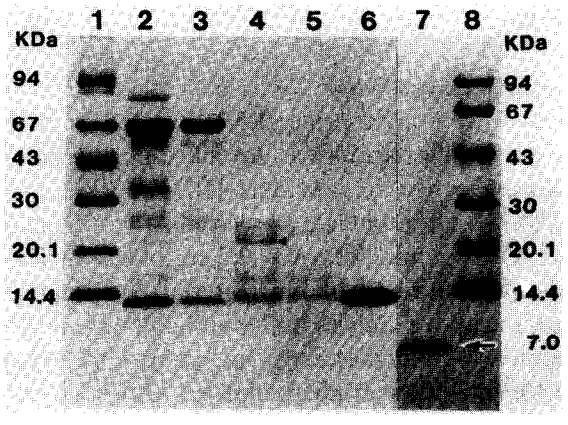

Fig. 1. SDS-PAGE of UP1-containing fractions at each step in procedure 1. Molecular mass markers or UP1-containing fractions at each step were electrophoresed either under non-reducing (lanes 1–6) or reducing (lanes 7 and 8) conditions as follows; molecular mass markers (lanes 1 and 8) and UP1-containing fractions at ammonium sulphate precipitation (lane 2), ConA affinity chromatography (lane 3), Sephadex G-75 column chromatography (lane 4), anti-RBP immunoaffinity chromatography (lane 5) and Mono Q column chromatography (lanes 6 and 7). Monomeric UP1 and RBP are indicated by an arrow and a filled triangle, respectively.

RBP was detected even in 640-fold dilution of this fraction by Western blotting analysis, whose minimum detection level was 0.625 μg/ml (Fig. 2, lanes 3–10).

Preliminary experiments revealed that both UP1 and RBP co-migrated on anion-exchange chromatography using a Mono Q column, probably owing to their similar p*I* values. We employed anti-RBP affinity chromatography to remove RBP before application to the Mono Q column. The band corresponding to RBP disappeared on SDS-PAGE analysis after this treatment (Fig. 1, lane 5). Furthermore this fraction showed no immunoreactivity on Western blotting analysis using the anti-RBP Ab (Fig. 2, lane 11), indicating that this procedure effectively removed RBP.

UP1 was eluted as a single peak from the Mono Q column (Fig. 3, indicated by a stippled bar). When 1 μg of this fraction was used for the Western blotting, no reactivity against anti-RBP Ab was observed (Fig. 2, lane 12), again indicating that RBP was completely removed from this fraction.

SDS-PAGE analysis showed that a single band was observed at 14 000 daltons under non-reducing conditions (Fig. 1, lane 6) and at 7000 daltons under reducing conditions (Fig. 1, lane 7). Furthermore, rabbit polyclonal anti-UP1 Ab detected these bands on Western blots (data not shown). These results indicated that UP1 is a homodimer consisting of two suburits of 7000 daltons bound by disulphide bridges, which agrees with a previous report [16]. Although the final yield of UP1 was 41.6% (Table I), this purification procedure is cumbersome. Hence, we tried to establish a simpler purification procedure using an anti-UP1 immunoaffinity gel.

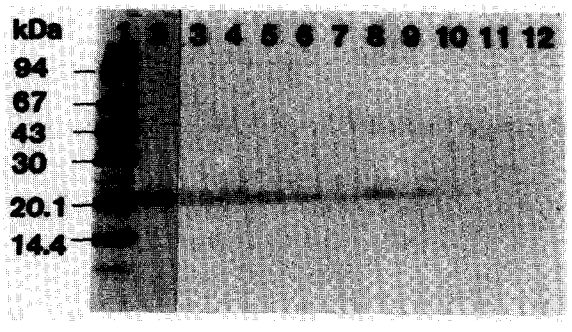

Fig. 2. Western blotting using anti-RBP Ab. A 10-μl volume of the samples was electrophoresed under non-reducing conditions as follows; molecular mass markers (lane 1), 50, 10, 5, 2.5, 1.25 or 0.625 μg/ml RBP (lanes 2–7), 160-, 320- or 640-fold dilution of G-75 fraction (lanes 8–10), anti-RBP fraction (lane 11) and 100 μg/ml purified UP1 (lane 12), respectively. Samples were then transferred onto a nitrocellulose membrane. The membrane was either stained with Amido Black 10B (lanes 1 and 2) or immunostained with anti-RBP Ab (lanes 3–11) as described under Experimental.

Specificity of the monoclonal and polyclonal anti-UP1 Ab

We prepared an anti-UP1 mAb in order to make an immunoaffinity gel and to simplify the purification procedure. We obtained one mAb clone, whose subclass was IgG1 kappa. Western blotting analysis revealed that the mAb detected 14 000 dalton UP1 under non-reducing conditions and 7000 dalton UP1 under reducing conditions (Fig. 4A).

Both monoclonal and polyclonal anti-UP1 Abs reacted with only UP1 among proteins from the starting ammonium sulphate fraction of the urine, as revealed by Western blotting analysis, indicating that these Abs were very specific for UP1 (Fig. 4B, lanes 2 and 3).

Purification procedure 2

We then purified UP1 by a new procedure using an anti-UP1 immunoaffinity gel. After Sephadex A-25 column chromatography, many contaminating proteins remained (Fig. 5, lane 3). These proteins were removed by immunoaffinity chromatography as judged by SDS-PAGE, which revealed a single band at 14 000 daltons (Fig. 5, lane 4). The fraction purified by the immunoaffinity gel was applied to reversed-phase HPLC. A single peak appeared at a concentration of *ca.* 60% acetonitrile (Fig. 6) and this fraction showed a single band at 14 000 daltons under non-reducing conditions on SDS-PAGE (Fig. 5, lane 5). No other major peaks, except for UP1, appeared on reversed-phase HPLC, indicating that the immunoaffinity chromatography was very effective for the purification of UP1.

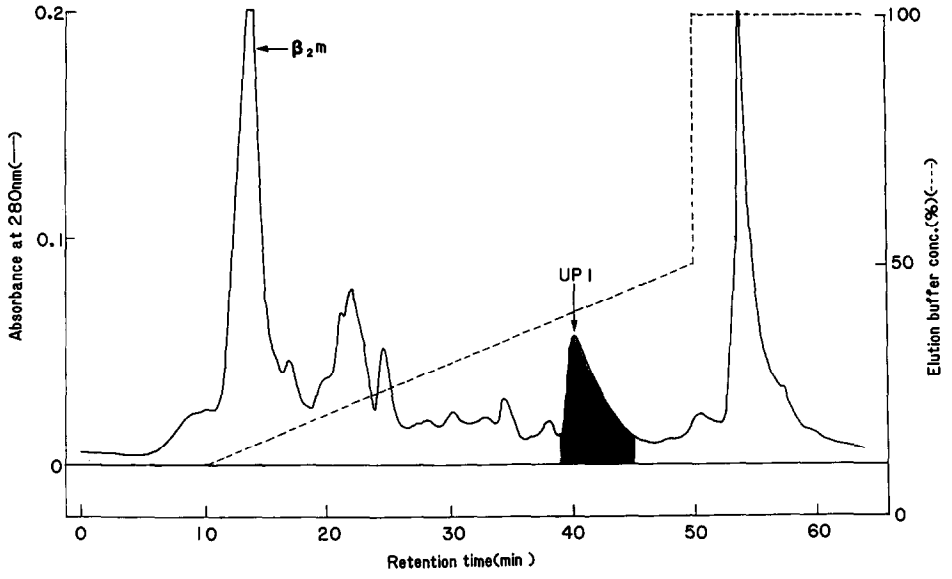

Fig. 3. Elution profile of Mono Q anion-exchange chromatography. UP1, a single peak, and β_2m were eluted at elution buffer concentration of 40 or 15%, respectively.

Furthermore, the final yield was 60.2% (Table I), which was much higher than that of purification procedure 1.

ELISA for UP1

When purified UP1 was used as a standard, the calibration curve of UP1 was established over the range 10–10 000 pg/ml (Fig. 7). The actual working range was 50–5000 pg/ml, and the intra-assay coefficients of variation for the standards were calculated to be 1.7–4.2% in this range. This ELISA is very specific for UP1 since other urinary proteins such as human IgG, human albumin, RBP, α_1m and β_2m did not show any cross-reactivity even at the highest concentration (100 μg/ml) we employed (data not shown).

TABLE I

YIELD OF UP1 AT EACH PURIFICATION STEP

Total protein was determined using a Coomassie protein assay reagent (Pierce). UP1 concentration was determined by an ELISA for UP1 as described under Experimental.

Purification step	Total protein (mg)	Total UP1 (μg)	Yield of UP1 (%)	Concentration (fold)
Procedure 1				
$(NH_4)_2SO_4$ precipitation	365.6	966.4	100.0	1
ConA affinity	204.8	922.9	95.5	1.7
Sephadex G-75	6.2	612.2	63.3	87.3
Anti-RBP affinity	2.7	486.9	50.3	68.2
Mono Q	0.4	402.6	41.6	380.7
Procedure 2				
$(NH_4)_2SO_4$ precipitation	750.0	904.8	100.0	1
Sephadex A-25	260.8	859.2	94.9	2.7
Anti-UP1 affinity	0.60	575.2	63.4	794.6
Reversed-phase HPLC	0.54	544.8	60.2	836.2

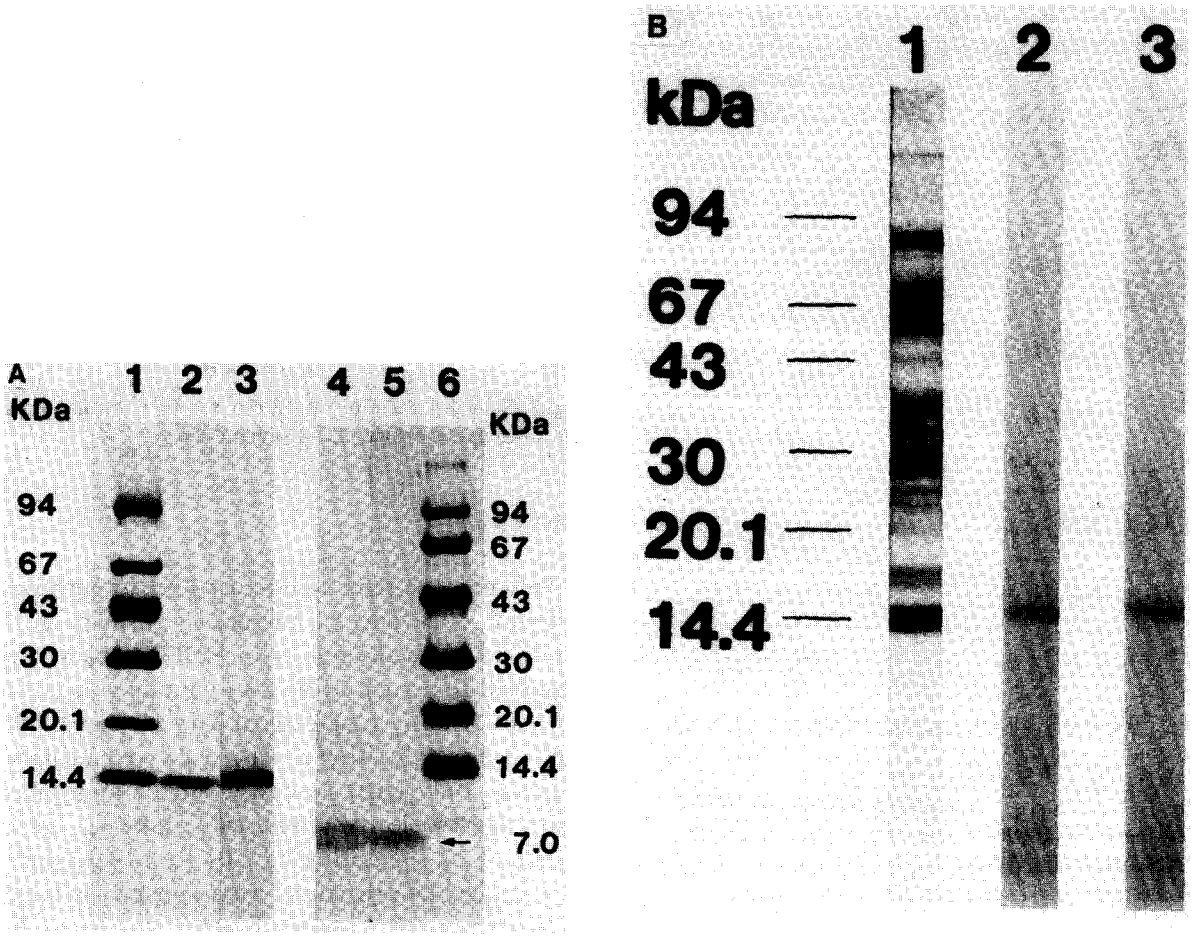

Fig. 4. Specificity of monoclonal and polyclonal anti-UP1 Ab. (A) Molecular mass markers (lanes 1 and 6) and 10 μg of purified UP1 (lanes 2–5) were electrophoresed under either non-reducing (lanes 1–3) or reducing (lanes 4–6) conditions and proteins were transferred onto a nitrocellulose membrane. The membrane was either stained with Amido Black 10B (lanes 1, 2, 5 and 6) or immunostained using an mAb against UP1 (lanes 3 and 4) as described under Experimental. Monomeric UP1 is indicated by an arrow. (B) A 10-μl volume of ammonium sulphate precipitation fraction (30 μg per lane) was electrophoresed under non-reducing conditions and transferred onto a membrane. The membrane was either stained with Amido Black 10B (lane 1) or immunostained with monoclonal (lane 2) or polyclonal (lane 3) anti-UP1 Ab.

Amino acid sequence of UP1

Jackson *et al.* [16] previously reported the partial amino acid sequence of UP1 from the N-terminal to the 47th residue (Fig. 8, row 3), which was almost identical with that of mature hCC10 protein, as deduced from its cDNA sequence (Fig. 8, row 1). The only difference, however, is at the N-terminal residue of UP1 (Fig. 8). We analysed the amino acid sequence of UP1. When *ca.* 1500 pmol of UP1 were loaded on the sequencer, the theoretical initial yield was 1406 pmol and the average amino acid repetitive yield was 89.6%.

The partial amino acid sequence of UP1 up to residue 53 was determined and is shown in Fig. 8, row 2. The first residue was identified as glutamic acid (Fig. 9), which is identical with that of mature hCC10. Furthermore, the amino acid sequence of UP1 was identical with that of hCC10 up to the 53rd residue. These results indicate that UP1 and mature hCC10 are identical. Although no signal was recognized in the third residue (x, Fig. 8, row 2), it would be cysteine as deduced from cDNA sequence of hCC10.

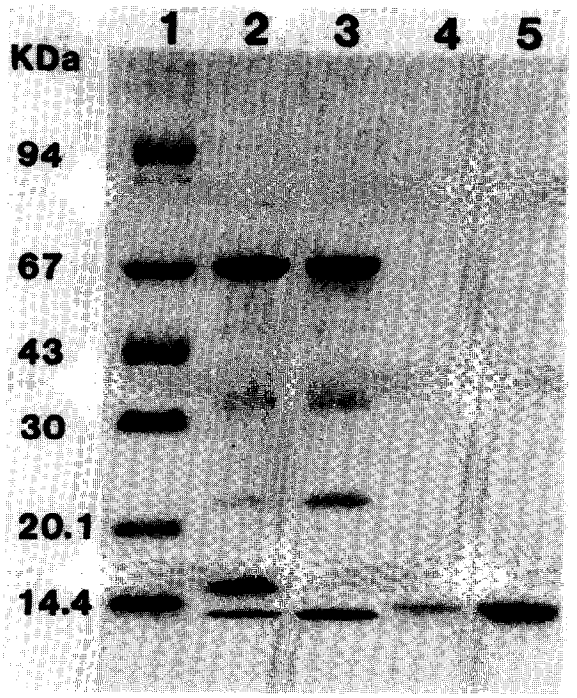

Fig. 5. SDS-PAGE of UP1-containing fractions at each step in procedure 2. SDS-PAGE was performed under non-reducing conditions on UP1-containing fractions after ammonium sulphate precipitation (lane 2), Sephadex A-25 column chromatography (lane 3), anti-UP1 immunoaffinity chromatography (lane 4) or reversed-phase HPLC (lane 5). Molecular mass markers were electrophoresed under the same conditions (lane 1).

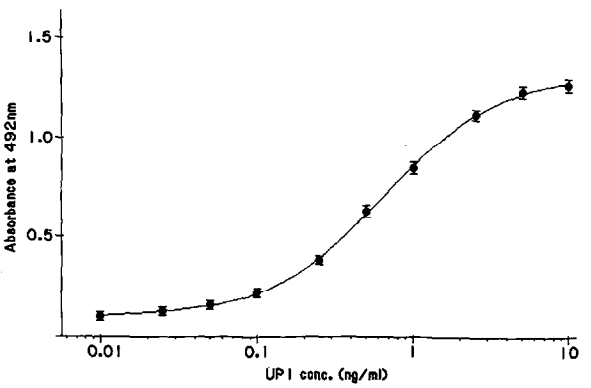

Fig. 7. Standard curve of the ELISA for UP1. Mean values (closed circles, $n = 8$) ± S.D. (bars) are shown.

DISCUSSION

Patients with renal failure usually excrete large amounts of several plasma-derived urinary proteins, including albumin, α_1m, RBP and β_2m. We observed that such urine also contained a large amount of UP1, in agreement with Jackson *et al.* [16]. Among the above plasma-derived urinary proteins, only RBP showed a similar molecular mass and isoelectric point with those of UP1, thus making separation of UP1 from RBP difficult. In the first procedure, we performed an anti-RBP affinity chromatography prior to a Mono Q

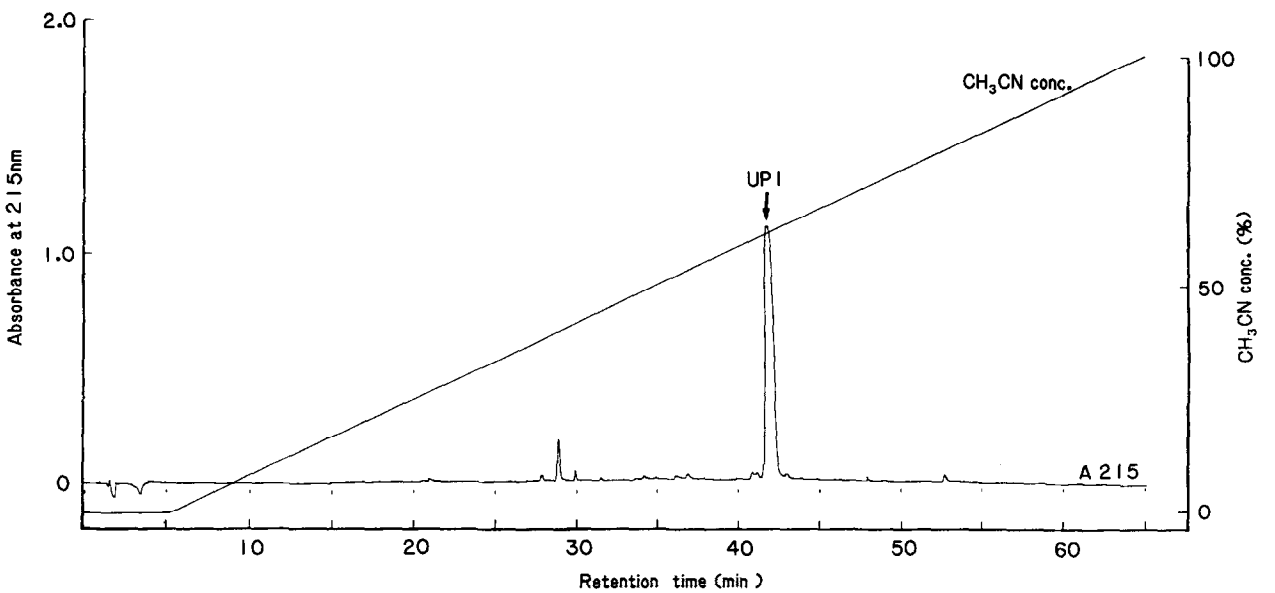

Fig. 6. Elution profile of reversed-phase HPLC. The position of the UP1-containing fraction is indicated by an arrow.

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                    -21
hCC10               Met Lys Leu Ala Val Thr Leu Thr Leu Val

                        -10
hCC10               Thr Leu Ala Leu Cys Cys Ser Ser Ala Ser

                          1
hCC10               Ala Glu Ile Cys Pro Ser Phe Gln Arg Val

UP1(this study)         Glu Ile  x  Pro Ser Phe Gln Arg Val

UP1(Jackson et al.)     Gly Ile Cys Pro Ser Phe Gln Arg Val

                     10
hCC10               Ile Glu Thr Leu Leu Met Asp Thr Pro Ser

UP1(this study)     Ile Glu Thr Leu Leu Met Asp Thr Pro Ser

UP1(Jackson et al.) Ile Glu Thr Leu Leu Met Asp Thr Pro Ser

                     20
hCC10               Ser Tyr Glu Ala Ala Met Glu Leu Phe Ser

UP1(this study)     Ser Tyr Glu Ala Ala Met Glu Leu Phe Ser

UP1(Jackson et al.) Ser Tyr Glu Ala Ala Met Glu Leu Phe Ser

                     30
hCC10               Pro Asp Gln Asp Met Arg Glu Ala Gly Ala

UP1(this study)     Pro Asp Gln Asp Met Arg Glu Ala Gly Ala

UP1(Jackson et al.) Pro Asp Gln Asp Met Arg Glu Ala Gly Ala

                     40
hCC10               Gln Leu Lys Lys Leu Val Asp Thr Leu Pro

UP1(this study)     Gln Leu Lys Lys Leu Val Asp Thr Leu Pro

UP1(Jackson et al.) Gln Leu Lys Lys Leu Val Asp Thr --- ---

                     50
hCC10               Gln Lys Pro Arg Glu Ser Ile Ile Lys Leu

UP1(this study)     Gln Lys Pro Arg --- --- --- --- --- ---

UP1(Jackson et al.) --- --- --- --- --- --- --- --- --- ---

                     60                                  70
hCC10               Met Glu Lys Ile Ala Gln Ser Ser Leu Cys Asn

UP1(this study)     --- --- --- --- --- --- --- --- --- --- ---

UP1(Jackson et al.) --- --- --- --- --- --- --- --- --- --- ---
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Fig. 8. Amino acid sequences of hCC10 and UP1. Row 1 shows the amino acid sequence of hCC10 deduced from its cDNA sequence. The first residue of mature hCC10 is indicated by the number 1. Rows 2 and 3 show the partial amino acid sequence of UP1 that we analysed and that of UP1 reported by Jackson *et al.* [16], respectivley. The cross in row 2, residue 3, indicates an unidentified amino acid from the HPLC analysis. Other unidentified residues are indicated by broken lines.

anion-exchange chromatography. This procedure effectively removed RBP from samples, as revealed by SDS-PAGE analysis. In order to simplify the purification procedure, we prepared an anti-UP1 immunoaffinity gel using the mAb against UP1. This immunoaffinity chromatography alone concentrated UP1 *ca.* 290-fold, with complete elimination of RBP from samples. Furthermore, both anti-RBP and anti-UP1 immunoaffinity gels were stable even after they were

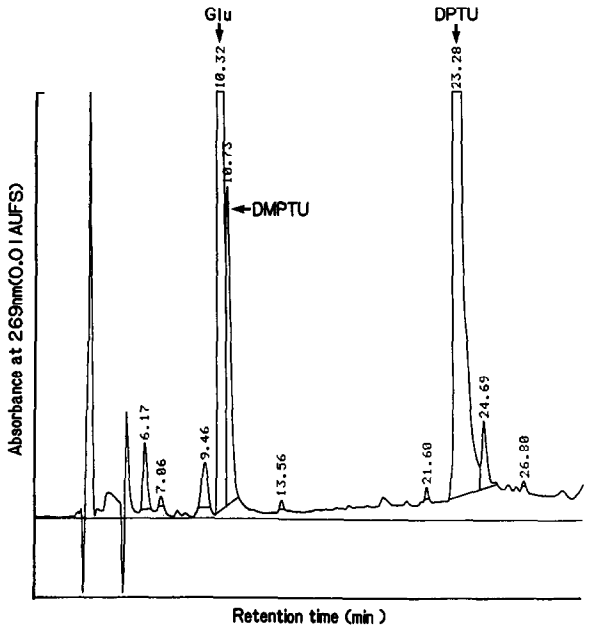

Fig. 9. Chromatogram of the N-terminal residue analysis. The N-terminal residue was identified as glutamic acid (indicated by Glu at a retention time of 10.32 min). The positions of dimethylphenylthiourea (DMPTU) and diphenylthiourea (DPTU) are also indicated.

used more than ten times. These results suggest that such immunoaffinity chromatography is very effective in differentiating a protein that is contaminated with other proteins with similar physicochemical properties.

We found that the amino acid sequence of UP1 is identical with that of mature hCC10 up to the 53rd residue and we demonstrated that N-terminal residue is glutamic acid, not glycine as reported by Jackson *et al.* [16]. Because we purified UP1 from the pooled urine of three unrelated patients with chronic renal failure and observed only a single peak identified as glutamic acid on the N-terminal analysis, it is unlikely that the difference at the N-terminal residue was due to genetic polymorphism. The codon encoding the first amino acid of mature hCC10 is GAG [15]. Therefore, we cannot completely rule out that some individuals have a single-base substitution from GAG to GGG, which may result in a change from glutamic acid to glycine.

Of note is that UP1/hCC10 has 61% homology in amino acid sequence with rabbit uteroglobin [15]. Singh *et al.* [11] compared the biological properties of hCC10 with those of rabbit uteroglobin. Both proteins inhibited pancreatic phospholipase A_2 activity. In addition, rabbit uteroglobin also inhibited macrophage phospholipase A_2 activity [10]. Although rabbit uteroglobin inhibited papain and bound progesterone, hCC10 did not inhibit papain and had only a limited capacity to bind progesterone. Furthermore, rabbit uteroglobin has been suggested to prevent maternal immunological reactions against the developing conceptus in utero [12] and also to mask the immunogenicity of male gametes in the female genital tract following coitus [13]. Although UP1/hCC10 has a high degree of homology with rabbit uteroglobin, there are few reports on the immunosuppressive properties of this protein. Therefore, the mAb that we prepared may be useful in analysing the biological properties of UP1/hCC10.

Recently, urinary levels of UP1 have been measured with a latex immunoassay or ELISA [23–25]. The excretion of urinary UP1 is elevated in the diabetic [23] and in cadmium nephropathy [24], indicating that UP1 will become a clinical indicator for the renal dysfunction as well as other urinary proteins such as β_2m [21], α_1m [22] and RBP [26]. Using an ELISA for UP1, we also observed that normal males excreted more UP1 into the urine than normal females after puberty as reported previously [23]. Simultaneous determination of serum, however, revealed that there was no sex difference in serum UP1 levels even after adolescence (our unpublished data). Recently, uteroglobin-like proteins were detected immunohistochemically in the human uterus [27,28] and prostate [29] in addition to the lung tissues [30]. The significance of the sex difference in urine levels of UP1 will be clarified by the determination of UP1/hCC10 levels in various body fluids.

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